

Fc fusion proteins of human erythropoietin with high biological activities

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Background

[0001] Erythropoietin (EPO) is a 30.4 kilodalton (kDa) glycoprotein hormone that promotes the proliferation of erythroid progenitor cells and supports their differentiation into mature erythrocytes (see, for example, Krantz, *Blood*, 77:419-434, 1991). EPO is produced in the adult kidney and the fetal liver. In adults, EPO is produced primarily in kidney cells in response to hypoxia or anemia and circulates in the bloodstream. EPO targets the 66 kDa specific receptor (EPO-Rc) found almost exclusively on the surface of erythroid progenitor cells present in bone marrow. Upon binding EPO, the receptor is activated and undergoes homodimerization, followed by tyrosine phosphorylation. Subsequently, a series of intracellular signal transduction events take place, leading to the increase of the number of the progenitor cells and their maturation into erythrocytes (see, for example, Lodish et al., *Cold Spring Harbor Symp. Quant. Biol.*, 60:93-104, 1995).

[0002] Recombinant human EPO (rHuEPO) is widely used in the treatment of patients with chronic anemia due to renal diseases at both end-stage and pre-dialysis phases. Administration of EPO has also been successful to treat anemia in patients caused by cancer chemotherapy, rheumatoid arthritis, AZT treatment for HIV infection and myelodysplastic syndrome. No direct toxic effect of treatment has been reported and the benefits of blood transfusion could be achieved without the transfusion.

[0003] The concentration of EPO in normal human serum varies approximately from 0.01 to 0.03 units/ml. Supplemental EPO is a desirable treatment in cases of renal failure with decreased EPO production. The half-life for the serum clearance of intravenous (i.v.) rHuEPO is approximately 4 to 13 h. The peak serum concentration for subcutaneous (s.c.) rHuEPO occurs in 5 to 24 h after injection with an elimination half-life

of 17 h. The s.c. administration route can therefore lead to much longer retention in the blood than i.v. administration of the same dose. The mechanism responsible for clearing EPO from the serum remains unclear. In animal experiments, less than 5% is excreted by the kidney. The liver, which rapidly removes asialated EPO, has not been shown to play a significant role in clearing EPO (see, for example, Fried, *Annu. Rev. Nutr.*, 15:353-377, 1995).

[0004] Immunoglobulins of IgG class are among the most abundant proteins in human blood. Their circulation half-lives can reach as long as 21 days. Fusion proteins have been reported to combine the Fc regions of IgG with the domains of another protein, such as various cytokines and soluble receptors (see, for example, Capon et al., *Nature*, 337:525-531, 1989; Chamow et al., *Trends Biotechnol.*, 14:52-60, 1996); US patents 5,116,964 and 5,541,087). The prototype fusion protein is a homodimeric protein linked through cysteine residues in the hinge region of IgG Fc, resulting in a molecule similar to an IgG molecule without the CH1 domains and light chains. Due to the structural homology, Fc fusion proteins exhibit *in vivo* pharmacokinetic profile comparable to that of human IgG with a similar isotype. This approach has been applied to several therapeutically important cytokines, such as IL-2 and IFN- α_{2a} , and soluble receptors, such as TNF-Rc and IL-5-Rc (see, for example, US patents 5,349,053 and 6,224,867). To extend the circulating half-life of EPO and/or to increase its biological activity, it is desirable to make fusion proteins containing EPO linked to the Fc portion of the human IgG protein as disclosed or described in this invention.

[0005] In most of the reported Fc fusion protein molecules, a hinge region serves as a spacer between the Fc region and the cytokine or soluble receptor at the amino-terminus, allowing these two parts of the molecule to function separately (see, for example, Ashkenazi et al., *Current Opinion in Immunology*, 9:195-200, 1997). Relative to the EPO monomer, a fusion protein consisting of two complete EPO domains separated by a 3- to 7-amino acid peptide linker exhibited reduced activity (Qiu et al., *J. Biol. Chem.*, 273:11173-11176, 1998). However, when the peptide linker between the two EPO domains was 17 amino acids in length, the dimeric EPO molecule exhibited considerably enhanced *in vitro*

and *in vivo* activities. The enhanced activity has been shown to be due to an increased *in vitro* activity coupled with a different pharmacokinetic profile in mice (see, for example, Sytkowski et al., *J. Biol. Chem.*, 274:24773-24778, 1999; US patent 6,187,564). It is discovered according to this invention that an added peptide linker present between HuEPO and a human IgG Fc variant renders the *in vitro* biological activity of the HuEPO-L-Fc molecule high. A human EPO fusion protein with an appropriate peptide linker between the HuEPO and Fc moieties (HuEPO-L-Fc) exhibits an *in vitro* biological activity similar to or higher than that of rHuEPO itself on a molar basis. While not bound by any theory, it is believed that this "similar to or higher than" *in vitro* biological activity is achieved in two ways: (1) keeping the Fc region away from the EPO-Rc binding sites on EPO, and (2) keeping one EPO from the other EPO domain, so both EPO domains can interact with EPO-Rc on the erythroid progenitor cells independently. For the present invention, a flexible peptide linker of about 20 or fewer amino acids in length is preferred. It is preferably to use a peptide linker comprising of two or more of the following amino acids: glycine, serine, alanine, and threonine.

[0006] The Fc region of human immunoglobulins plays a significant role in immune defense for the elimination of pathogens. Effector functions of IgG are mediated by the Fc region through two major mechanisms: (1) binding to the cell surface Fc receptors (Fc γ Rs) can lead to ingestion of pathogens by phagocytosis or lysis by killer cells *via* the antibody-dependent cellular cytotoxicity (ADCC) pathway, or (2) binding to the C1q part of the first complement component C1 initiates the complement-dependent cytotoxicity (CDC) pathway, resulting in the lysis of pathogens. Among the four human IgG isotypes, IgG1 and IgG3 are effective in binding to Fc γ R. The binding affinity of IgG4 to Fc γ R is an order of magnitude lower than that of IgG1 or IgG3, while binding of IgG2 to Fc γ R is below detection. Human IgG1 and IgG3 are also effective in binding to C1q and activating the complement cascade. Human IgG2 fixes complement poorly, and IgG4 appears quite deficient in the ability to activate the complement cascade (see, for example, Jefferis et al., *Immunol. Rev.*, 163:59-76, 1998). For therapeutic use in humans, it is essential that when HuEPO-L-Fc binds to EPO-Rc on the surface of the erythroid progenitor cells, the Fc region

of the fusion protein will not mediate undesirable effector functions, leading to the lysis or removal of these progenitor cells. Accordingly, the Fc region of HuEPO-L-Fc must be of a non-lytic nature, *i. e.* the Fc region must be inert in terms of binding to Fc γ Rs and C1q for the triggering of effector functions. It is clear that none of the naturally occurring IgG isotypes is suitable for use to produce the HuEPO-L-Fc fusion protein. To obtain a non-lytic Fc, certain amino acids of the natural Fc region have to be mutated for the attenuation of the effector functions.

[0007] By comparing amino acid sequences of human and murine IgG isotypes, a portion of Fc near the N-terminal end of the CH2 domain is implicated to play a role in the binding of IgG Fc to Fc γ Rs. The importance of a motif at positions 234 to 237 has been demonstrated using genetically engineered antibodies (see, for example, Duncan et al., *Nature*, 332:563-564, 1988). The numbering of the amino acid residues is according to the EU index as described in Kabat et al. (in *Sequences of Proteins of Immunological Interest*, 5th Edition, United States Department of Health and Human Services, 1991). Among the four human IgG isotypes, IgG1 and IgG3 bind Fc γ Rs the best and share the sequence Leu234-Leu-Gly-Gly237 (only IgG1 is shown in Figure 1). In IgG4, which binds Fc γ Rs with a lower affinity, this sequence contains a single amino acid substitution, Phe for Leu at position 234. In IgG2, which does not bind Fc γ Rs, there are two substitutions and a deletion leading to Val234-Ala-Gly237 (Figure 1). To minimize the binding of Fc to Fc γ R and hence the ADCC activity, Leu235 in IgG4 has been replaced by Ala (see, for example, Hutchins et al., *Proc. Natl. Acad. Sci. USA*, 92:11980-11984, 1995). IgG1 has been altered in this motif by replacing Glu233-Leu-Leu235 with Pro233-Val-Ala235, which is the sequence from IgG2. This substitution resulted in an IgG1 variant devoid of Fc γ R-mediated ability to deplete target cells in mice (see, for example, Isaacs et al., *J. Immunol.*, 161: 3862-3869, 1998).

[0008] A second portion that appears to be important for both Fc γ R and C1q binding is located near the carboxyl-terminal end of CH2 domain of human IgG (see, for example, Duncan et al., *Nature*, 332:738-740, 1988). Among the four human IgG isotypes,

there is only one site within this portion that shows substitutions: Ser330 and Ser331 in IgG4 replacing Ala330 and Pro331 present in IgG1, IgG2, and IgG3 (Figure 1). The presence of Ser330 does not affect the binding to Fc γ R or C1q. The replacement of Pro331 in IgG1 by Ser virtually abolished IgG1 ability to C1q binding, while the replacement of Ser331 by Pro partially restored the complement fixation activity of IgG4 (see, for example, Tao et al., *J. Exp. Med.*, 178:661-667, 1993; Xu et al., *J. Biol. Chem.*, 269:3469-3474, 1994).

[0009] We discover that at least three Fc variants (vFc) can be designed for the production of HuEPO-L-vFc fusion proteins (Figure 1). Human IgG2 Fc does not bind Fc γ R but showed weak complement activity. An Fc γ 2 variant with Pro331Ser mutation should have less complement activity than natural Fc γ 2 while remain as a non-binder to Fc γ R. IgG4 Fc is deficient in activating the complement cascade, and its binding affinity to Fc γ R is about an order of magnitude lower than that of the most active isotype, IgG1. An Fc γ 4 variant with Leu235Ala mutation should exhibit minimal effector functions as compared to the natural Fc γ 4. The Fc γ 1 variant with Leu234Val, Leu235Ala and Pro331Ser mutations also will exhibit much less effector functions than the natural Fc γ 1. These Fc variants are more suitable for the preparation of the EPO fusion proteins than naturally occurring human IgG Fc. It is possible that other replacements can be introduced for the preparation of a non-lytic Fc without compromising the circulating half-life or causing any undesirable conformational changes.

[0010] There are many advantages with the present invention. The high activity and prolonged presence of the HuEPO-L-vFc fusion protein in the serum can lead to lower dosages as well as less frequent injections. Less fluctuations of the drug in serum concentrations also means improved safety and tolerability. Less frequent injections may result in better patient compliance and quality of life. The HuEPO-L-vFc fusion protein containing a non-lytic Fc variant will therefore contribute significantly to the management of anemia caused by conditions including renal failure, cancer chemotherapy, rheumatoid arthritis, AZT treatment for HIV infection, and myelodysplastic syndrome.

Summary of the invention

[0011] One aspect of the present invention relates to a HuEPO-L-vFc fusion protein. The HuEPO-L-vFc fusion protein comprises HuEPO, a peptide linker, and a human IgG Fc variant. It is preferably to use a flexible peptide linker of 20 or fewer amino acids in length which comprises of two or more of the following amino acids: glycine, serine, alanine, and threonine. The IgG Fc variant is of non-lytic nature and contains amino acid mutations as compared to naturally occurring IgG Fc.

[0012] It is another embodiment of the present invention that the human Ig Fc comprises a hinge, CH2, and CH3 domains of human IgG, such as human IgG1, IgG2, and IgG4. The CH2 domain contains amino acid mutations at positions 228, 234, 235, and 331 (defined by the EU numbering system) to attenuate the effector functions of Fc.

[0013] In yet another embodiment of the present invention, a method is disclosed to make or produce such fusion proteins from a mammalian cell line such as a CHO-derived cell line. Growing transfected cell lines under conditions such that the recombinant fusion protein is expressed in its growth medium in excess of 10, preferably 30, µg per million cells in a 24 hour period. These HuEPO-L-vFc fusion proteins exhibit an *in vitro* biological activity similar to or higher than that of rHuEPO on a molar basis, and more preferably an extended serum half-life without undesirable side effects, leading to improved pharmacokinetics and pharmacodynamics, thus lower dosages and fewer injections would be needed to achieve similar efficacies.

Brief descriptions of the drawings

[0014] Figure 1 shows the amino acid sequence alignment from the hinge and CH2 regions of human IgG1, IgG2, IgG4 and their variants. Three portions are compared: amino acid position 228, 234-237, and 330-331. Amino acid mutations of the

variants are indicated in bold italics. The EU numbering system is used for the amino acid residues.

[0015] Figure 2 shows the nucleotide sequence and deduced amino acid sequence of (A) HuEPO-L-vFc_{γ2}, (B) HuEPO-L-vFc_{γ4}, and (C) HuEPO-L-vFc_{γ1} as the *HindIII-EcoRI* fragment in the respective pEFP expression vector. The peptide from amino acid residues -27 to -1 is the leader peptide of human EPO. The mature protein contains human EPO (amino acid residues 1 to 165), a peptide linker (amino acid residues 166 to 181), and a Fc variant (amino acid residues 182 to 409 of vFc_{γ2}, 182 to 410 of vFc_{γ4}, and 182 to 408 of vFc_{γ1}). In the Fc regions, nucleotide and corresponding amino acid mutations in bold are also underlined.

[0016] Figure 3 shows the effect of the purified proteins, HuEPO-L-vFc_{γ2} or rHuEPO, on the proliferation of the 32D1.9 cells.

[0017] Figure 4 shows the effect of the purified HuEPO-L-vFc_{γ2} protein on the red blood cell counts in rats.

[0018] Figure 5 shows the effect of the purified HuEPO-L-vFc_{γ2} protein on the hemoglobin values in rats.

[0019] Figure 6 shows circulating levels of HuEPO-L-vFc_{γ2} in rats after i.v. injections of the fusion protein.

Detailed description of the invention

1. Construction of the gene encoding the fusion proteins

1.1 Construction of the gene encoding the HuEPO-L-vFc_{γ2} fusion protein

[0020] A fusion protein is assembled from several DNA segments. To obtain the gene encoding the leader peptide and mature protein of human EPO, cDNA library of human fetal liver or kidney (obtained from Invitrogen, Carlsbad, CA) is used as the template in polymerase chain reaction (PCR). For the convenience of cloning, SEQ ID NO:1 (Table 1), which incorporates a restriction enzyme cleavage site (*Hind*III) is used as the 5' oligonucleotide primer. Table 1 shows the sequences of oligonucleotides used for the cloning of the HuEPO-L-vFc fusion proteins. The 3' primer (SEQ ID NO:2) eliminates the EPO termination codon and incorporates a *Bam*HI site. The resulting DNA fragments of approximately 600 bp in length are inserted into a holding vector such as pUC19 at the *Hind*III and *Bam*HI sites to give the pEPO plasmid. The sequence of the human EPO gene is confirmed by DNA sequencing.

Table 1. Sequences of Oligonucleotides.

SEQ ID NO:1

5'-cccaagccttggcgcgagatgggggtgca-3'

SEQ ID NO:2

5'-cggatccgtcccctgtcctgcaggcct-3'

SEQ ID NO:3

5'-gagcgcaaattgttggtgcga-3'

SEQ ID NO:4

5'-ggaattctcattttacccggagacaggga-3'

SEQ ID NO:5

5'-tggttttctcgatggaggctgggaggcct-3'

SEQ ID NO:6

5'-aggcctcccagcctccatcgagaaaacca-3'

SEQ ID NO:7

5'-cggatccggtggcggttccggtggaggcgggaagcggcggtggaggatcag
agcgcaaattgttggtgcga-3'

SEQ ID NO:8

5'-gagtccaaatatggtcccca-3'

SEQ ID NO:9

5'-ggaattctcatttaccagagacaggga-3'

SEQ ID NO:10

5'-cctgagttcgcggggggacca-3'

SEQ ID NO:11

5'-gagtccaaatatggtcccccattgccaccattgccagcacctgagttcgcgggggacca-3'

SEQ ID NO:12

5'-cggatccggtggcggttccggtggaggcggaagcggcggtggaggatcagagtccaaatatggtcccca-3'

SEQ ID NO:13

5'-gacaaaactcacacatgccca-3'

SEQ ID NO:14

5'-acctgaagtcgcggggggaccgt-3'

SEQ ID NO:15

5'-gacaaaactcacacatgccaccgtgccagcacctgaagtcgcggggggaccgt-3'

SEQ ID NO:16

5'-cggatccggtggcggttccggtggaggcggaagcggcggtggaggatcagacaaaactcacacatgccca-3'

[0021] The gene encoding the Fc region of human IgG2 (Fc_γ2) is obtained by reverse transcription and PCR using RNA prepared from human leukocytes and appropriate 5' (SEQ ID NO:3) and 3' (SEQ ID NO:4) primers. Resulting DNA fragments of Fc_γ2 containing complete sequences of the hinge, CH2, and CH3 domains of IgG2 will be used as the template to generate the Fc_γ2 Pro331Ser variant (vFc_γ2) in which Pro at position 331 of Fc_γ2 is replaced with Ser. To incorporate this mutation, two segments are produced and then assembled by using the natural Fc_γ2 as the template in overlapping PCR. The 5' segment is generated by using SEQ ID NO:3 as the 5' primer and SEQ ID NO:5 as the 3' primer. The 3' segment is generated by using SEQ ID NO:6 as the 5' primer and SEQ ID NO:4 as the 3' primer. These two segments are then joined at the region covering the Pro331Ser mutation by using SEQ ID NO:7 as the 5' primer and SEQ ID NO:4 as the 3' primer. The SEQ ID

NO:7 primer contains sequences encoding a 16-amino acid Gly-Ser peptide linker including a *Bam*HI restriction enzyme site. The resulting DNA fragments of approximately 700 bp in length are inserted into a holding vector such as pUC19 at the *Bam*HI and *Eco*RI sites to give the pL-vFc_γ2 plasmid. The sequence of the gene is confirmed by DNA sequencing.

[0022] To prepare the HuEPO-L-vFc_γ2 fusion gene, the EPO fragment is excised from the pEPO plasmid with *Hind*III and *Bam*HI and is purified by agarose gel electrophoresis. The purified fragment is then inserted to the 5'-end of the peptide linker in the pL-vFc_γ2 plasmid to give the pEPO-L-vFc_γ2 plasmid. The fusion gene comprises HuEPO, a Gly-Ser peptide linker and the Fc_γ2 variant gene.

[0023] The presence of a peptide linker between the EPO and Fc moieties increases the flexibility of the EPO domains and enhances its biological activity (see, for example, Sytkowski et al., *J. Biol. Chem.*, 274: 24773-8, 1999). For the present invention, a peptide linker of about 20 or fewer amino acids in length is preferred. Peptide linker comprising two or more of the following amino acids: glycine, serine, alanine, and threonine can be used. An example of the peptide linker contains Gly-Ser peptide building blocks, such as GlyGlyGlyGlySer. Figure 2A shows a fusion gene (SEQ ID NO: 17) containing sequences encoding HuEPO, a 16-amino acid peptide linker (GlySerGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySer, SEQ ID NO: 23), and the Fc_γ2 Pro331Ser variant, and its corresponding amino acid sequence (SEQ ID NO: 18).

[0024] The complete gene encoding the HuEPO-L-vFc fusion protein is then inserted at the *Hind*III and *Eco*RI sites of a mammalian expression vector, such as pcDNA3 (Invitrogen). The final expression vector plasmid, named pEFP2, contains the cytomegalovirus early gene promoter-enhancer which is required for high level expression in mammalian cells. The plasmid also contains selectable markers to confer ampicillin resistance in bacteria, and G418 resistance in mammalian cells. In addition, the pEFP2 expression vector contains the dihydrofolate reductase (DHFR) gene to enable the co-amplification of the HuEPO-L-vFc_γ2 fusion gene and the DHFR gene in the presence of

methotrexate (MTX) when the host cells are deficient in the DHFR gene expression (see, for example, US patent 4,399,216).

1.2 Construction of the gene encoding the HuEPO-L-vFc_{γ4} fusion protein

[0025] Human IgG4 is observed partly as half antibody molecules due to the dissociation of the inter-heavy chain disulfide bonds in the hinge domain. This is not seen in the other three human IgG isotypes. A single amino acid substitution replacing Ser228 with Pro, which is the residue found at this position in IgG1 and IgG2, leads to the formation of IgG4 complete antibody molecules (see, for example, Angal et al., *Molec. Immunol.*, 30:105-108, 1993; Owens et al., *Immunotechnology*, 3:107-116, 1997; US Patent 6,204,007). The Fc_{γ4} variant containing Leu235Ala mutation for the minimization of FcR binding will also give rise to a homogeneous fusion protein preparation with this additional Ser228Pro mutation.

[0026] The gene encoding the Fc region of human IgG4 (Fc_{γ4}) is obtained by reverse transcription and PCR using RNA prepared from human leukocytes and appropriate 5' primer (SEQ ID NO:8) and 3' primer (SEQ ID NO:9). Resulting DNA fragments of Fc_{γ4} containing complete sequences of the hinge, CH2, and CH3 domains of IgG4 is used as the template to generate the Fc_{γ4} variant with Ser228Pro and Leu235Ala mutations (vFc_{γ4}) in which Ser228 and Leu235 have been replaced with Pro and Ala, respectively. The CH2 and CH3 domains are amplified using the 3' primer (SEQ ID NO:9) and a 5' primer containing the Leu235Ala mutation (SEQ ID NO:10). This amplified fragment, together with a synthetic oligonucleotide of 60 bases in length (SEQ ID NO:11) containing both Ser228Pro and Leu235Ala mutations, are joined in PCR by using SEQ ID NO:12 as the 5' primer and SEQ ID NO:9 as the 3' primer. The SEQ ID NO:12 primer contains sequences encoding a 16-amino acid Gly-Ser peptide linker including the *Bam*HI site. The resulting DNA fragments of approximately 700 bp in length are inserted into a holding vector such as pUC19 at the *Bam*HI and *Eco*RI sites to give the pL-vFc_{γ4} plasmid. The sequence of the gene is confirmed by DNA sequencing.

[0027] To prepare the HuEPO-L-vFc_{γ4} fusion gene, the HuEPO fragment is excised from the pEPO plasmid with *Hind*III and *Bam*HI and then inserted to the 5'-end of the peptide linker in the pL-vFc_{γ4} plasmid to give the pEPO-L-vFc_{γ4} plasmid. This fusion gene comprising HuEPO, a 16-amino acid Gly-Ser peptide linker and the Fc_{γ4} variant gene is then inserted at the *Hind*III and *Eco*RI sites of a mammalian expression vector, such as pcDNA3 (Invitrogen), as described for the HuEPO-L-vFc_{γ2} fusion protein. The final expression vector plasmid is designated as pEFP4. Figure 2B shows a fusion gene (SEQ ID NO:19) containing sequences encoding HuEPO, a 16-amino acid peptide linker (GlySerGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySer, SEQ ID NO:23), and the Fc_{γ4} variant with Ser228Pro and Leu235Ala mutations, and its corresponding amino acid sequence (SEQ ID NO: 20).

1.3 Construction of the gene encoding the HuEPO-L-vFc_{γ1} fusion protein

[0028] The hinge domain of human IgG1 heavy chain contains 15 amino acid residues (GluProLysSerCysAspLysThrHisThrCysProProCysPro, SEQ ID NO:24) including three cysteine residues. Out of these 3 cysteine residues, the 2nd and 3rd are involved in the formation of disulfide bonding between two heavy chains. The 1st cysteine residue is involved in the disulfide bonding to the light chain of IgG. Since there is no light chain present in the Fc fusion protein molecule, this cysteine residue may pair with other cysteine residues, leading to nonspecific disulfide bonding. The hinge domain of Fc_{γ1} can be truncated to eliminate the 1st cysteine residue (AspLysThrHisThrCysProProCysPro, SEQ ID NO: 25). The gene encoding the Fc_{γ1} region is obtained by reverse transcription and PCR using RNA prepared from human leukocytes and appropriate 5' primer (SEQ ID NO:13) and 3' primer (SEQ ID NO:4). Resulting DNA fragments containing the truncated hinge and complete sequences of CH2 and CH3 domains of Fc_{γ1} is used as the template to generate the Fc_{γ1} variant with Leu234Val, Leu235Ala, and Pro331Ser mutations (vFc_{γ1}).

[0029] One way to incorporate these mutations is as follows: two segments are produced and then assembled by using the natural Fc_{γ1} as the template in overlapping

PCR. The 5' segment is generated by using SEQ ID NO:14 as the 5' primer and SEQ ID NO:5 as the 3' primer. This 5' primer contains the Leu234Val, Leu235Ala mutations and the 3' primer contains the Pro331Ser mutation. The 3' segment is generated by using SEQ ID NO:6 as the 5' primer and SEQ ID NO:4 as the 3' primer. These 5' and 3' segments are then joined at the region covering the Pro331Ser mutation by using SEQ ID NO:14 as the 5' primer and SEQ ID NO:4 as the 3' primer. This amplified fragment of approximately 650 bp in length, together with a synthetic oligonucleotide of 55 bases (SEQ ID NO:15) containing Leu234Val and Leu235Ala, are joined in PCR by using SEQ ID NO:16 as the 5' primer and SEQ ID NO:4 as the 3' primer. The SEQ ID NO:16 primer contains sequences encoding a 16-amino acid Gly-Ser peptide linker including the *Bam*HI site. The resulting DNA fragments of approximately 700 bp in length are inserted into a holding vector such as pUC19 at the *Bam*HI and *Eco*RI sites to give the pL-vFc_γ1 plasmid. The sequence of the gene is confirmed by DNA sequencing.

[0030] To prepare the HuEPO-L-vFc_γ1 fusion gene, the EPO fragment is excised from the pEPO plasmid with *Hind*III and *Bam*HI and inserted to the 5'-end of the peptide linker in the pL-vFc_γ1 plasmid to give the pEPO-L-vFc_γ1 plasmid. The fusion gene comprising HuEPO, a 16-amino acid Gly-Ser peptide linker, and the Fc_γ1 variant gene is then inserted at the *Hind*III and *Eco*RI sites of a mammalian expression vector, such as pcDNA3 (Invitrogen), as described for the HuEPO-L-vFc_γ2 fusion protein. The final expression vector plasmid is designated as pEFP1. Figure 2C shows a fusion gene (SEQ ID NO: 21) containing sequences encoding HuEPO, a 16-amino acid peptide linker (GlySerGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySer, SEQ ID NO: 23), and the Fc_γ1 variant with Leu234Val, Leu235Ala and Pro331Ser mutations, and its corresponding amino acid sequence (SEQ ID NO: 22).

2. Expression of the fusion protein in transfected cell lines

[0031] The recombinant pEFP1, pEFP2 or pEFP4 expression vector plasmid is transfected into a mammalian host cell line to achieve the expression of the HuEPO-L-vFc

fusion protein. For stable high levels of expression, a preferred host cell line is Chinese Hamster Ovary (CHO) cells deficient in the DHFR enzyme (see, for example, US patent 4,818,679). A preferred method of transfection is electroporation. Other methods, including calcium phosphate co-precipitation, lipofectin, and protoplast fusion, can also be used. For electroporation, 10 μ g of plasmid DNA linearized with *Bsp*CI is added to 2 to 5 $\times 10^7$ cells in a cuvette using Gene Pulser Electroporator (Bio-Rad Laboratories, Hercules, CA) set at an electric field of 250 V and a capacitance of 960 μ Fd. Two days following the transfection, the media are replaced with growth media containing 0.8 mg/ml of G418. Transfectants resistant to the selection drug are tested for the secretion of the fusion protein by anti-human IgG Fc ELISA. Quantitation of the expressed fusion protein can also be carried out by ELISA using anti-HuEPO assays. The wells producing high levels of the Fc fusion protein are subcloned by limiting dilutions on 96-well tissue culture plates.

[0032] To achieve higher levels of the fusion protein expression, co-amplification is preferred by utilizing the gene of DHFR which can be inhibited by the MTX drug. In growth media containing increasing concentrations of MTX, the transfected fusion protein gene is co-amplified with the DHFR gene. Transfectants capable of growing in media with up to 1 μ g/ml of MTX are again subcloned by limiting dilutions. The subcloned cell lines are further analyzed by measuring the secretion rates. Several cell lines yielding secretion rate levels over about 10 to about 30 μ g/ 10^6 cells/24h, are adapted to suspension culture using serum-free growth media. The conditioned media are then used for the purification of the fusion protein. One cell line, E1-6, secreting HuEPO-L-vFc $_{\gamma 2}$ at about 20 μ g/ 10^6 cells/24h was adapted to grow in suspension culture in 100-ml spinner flasks. After 10 days, cumulative concentration of the fusion protein reached approximately 200 μ g/ml as analyzed by quantitative ELISA using the purified HuEPO-L-vFc $_{\gamma 2}$ protein as the standards. A single-cell subclone of E1-6, designated E1-6-A18, exhibited a secretion rate of HuEPO-L-vFc $_{\gamma 2}$ at about 38 μ g/ 10^6 cells/24h.

[0033] Sugar side chain structures are crucial for the *in vivo* activity of EPO. The terminal sugar chain of the Asn-linked carbohydrate contains sialic acids, repeating

poly-N-acetyllactosamine and glucose. Recombinant HuEPO expressed in certain mammalian cells such as NS0 is known to give proteins with low sialic acid content. Removal of sialic acids, which leads to exposure of the penultimate galactose residues, increases the affinity for hepatic asialoglycoprotein binding lectin. This trapping pathway results in decrease of *in vivo* biological activity as measured in whole animals. Recombinant HuEPO produced in CHO cells exhibit glycosylation patterns very similar to that found in the natural EPO (see, for example, Takeuchi et al., *Proc. Natl. Acad. Sci. USA*, 86:7819-22, 1989). The HuEPO-L-vFc fusion proteins expressed and produced in accordance with this invention will show similar or higher biological activities when compared to rHuEPO on a molar basis.

3. Purification and characterization of the fusion protein

[0034] Conditioned media containing the fusion protein are titrated with 1 N NaOH to a pH of 7 to 8 and filtered through a 0.45 micron cellulose nitrate filter. The filtrate is loaded onto a Prosep A column equilibrated in phosphate-buffered saline (PBS). After binding of the fusion protein to Prosep A, the flow-through fractions are discarded. The column is washed with PBS until OD at 280 nm is below 0.01. The bound fusion protein is then eluted with 0.1 M citrate buffer at pH 3.75. After neutralizing with 0.4 volume of 1 M K₂HPO₄, fractions containing purified protein are pooled and dialyzed against PBS. The solution is then filtered through a 0.22 micron cellulose nitrate filter and stored at 4°C. The purified HuEPO-L-vFc_γ2 protein is quantitated by BCA protein assay using BSA as the standard. The molecular weight of the purified protein is in the range of 120 and 140 kDa by SDS-PAGE under non-reducing conditions. Under reducing conditions, the purified protein migrates around approximately 65 kDa. HPLC analysis did not show any appreciable aggregates in the purified HuEPO-L-vFc_γ2 protein.

4. In vitro biological assays

[0035] Supernatants of transfectants or purified proteins can be tested for their ability to stimulate the proliferation of the 32D1.9 cells. The 32D1.9 cell line is a

murine cell line that has been transfected with the human EPO-Rc gene. Like TF-1 cells (Kitamura et al., J. Cell. Physiol., 140:323-334, 1989), the 32D1.9 cells express human EPO-Rc on their cell surface and are responsive to EPO. The cells are maintained in growth medium (RPMI-1640 medium containing 10% FCS and human IL-5 at 1 to 5 ng/ml). Log phase 32D1.9 cells are collected and washed with assay medium (growth medium without human IL-5). A total of 1×10^4 cells per sample of 32D1.9 in 50 μ l is added to each well of a 96-well tissue culture plate. The cells are incubated with 50 μ l of assay media containing various concentrations of the HuEPO-L-vFc fusion protein or the rHuEPO control from 0.001 to 30 nM each. The plate is kept at 37°C and 5% CO₂ in a humidified incubator for 4 days before 10 μ l of Methylthiazolylphenyl-tetrazolium bromide (MTT, 2.5 mg/ml in PBS) is added to each well. After 4 h, the cells and formazan are solubilized by adding 100 μ l per well of 10% SDS in 0.01 N HCl. The plate is then read at 550 nm with the reference beam set at 690 nm. The OD reading is plotted against the concentration of rHuEPO or the fusion protein. The inflection point of the sigmoidal curve represents the concentration at which 50% of the maximal effect, ED₅₀, is induced. The biological activity of HuEPO-L-vFc relative to that of rHuEPO can therefore be compared quantitatively. Figure 3 shows the *in vitro* biological activity of the HuEPO-L-vFc₂ fusion protein. Varying concentrations of rHuEPO or HuEPO-L-vFc₂ were assayed for their abilities to stimulate the proliferation of the 32D1.9 cells. On a molar basis, HuEPO-L-vFc₂ exhibits similar activity as that of rHuEPO, about 3 to about 4×10^6 units/ μ mole.

[0036] Supernatants of transfectants or purified proteins can also be tested for their ability to stimulate the proliferation and differentiation of human bone marrow progenitor cells to form red blood cell colonies, colony forming unit-erythroid (CFU-E). The procedure is as follows. Light-density cells from human bone marrow centrifuged over Ficoll-Paque are washed and resuspended at 1×10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM) containing 5% FCS. These cells are incubated in a tissue culture dish overnight at 37°C and 5% CO₂ to remove all adherent cells including monocytes, macrophages, endothelial cell, and fibroblasts. Cells in suspension are then adjusted to 1×10^5 cells/ml in IMDM containing 5% FCS. For the assay, 0.3 ml of cells, 15

μl of stem cell factor at 20 μg/ml, 2.4 ml of methylcellulose, and 0.3 ml of media containing several concentrations of HuEPO-L-vFc (or rHuEPO control) are mixed. One ml of this cell mixture is plated on a 35-mm petri dish. The dishes are then kept at 37°C and 5% CO₂ for 10 to 14 d before the colonies are counted. A dose responsive curve can be plotted against the concentrations of HuEPO-L-vFc relative to those of rHuEPO.

5. In vivo pharmacokinetic studies in rats

5.1 Experimental protocol

[0037] One hundred male SD rats with an average body weight of about 200 g each were checked by retro-orbital bleeds for red blood cell counts (RBC) and hemoglobin values (Hb). Fifty-seven rats within a small range of RBC were put into 19 groups with 3 rats in each group. One group was designated as the control group to receive i.v. injection of 0.25 ml of PBS per kg weight. Remaining 18 groups received i.v. injection of HuEPO-L-vFc_{γ2} at 100 μg/kg. Blood samples were taken at different time points after injection: 1min, 2.5min, 5min, 10min, 20min, 1h, 2h, 4h, 1d, 2d, 3d, 4d, 5d, 6d, 7d, 8d, 9d, and 10d. One ml each of whole blood from days 2 to 10 was used to test for RBC and Hb. At all time points, 2 ml of whole blood was collected into tubes containing anticoagulant, cells were removed, and plasma was frozen at -70°C until assay was carried out.

5.2 The effect of i. v. injection of HuEPO-L-vFc_{γ2} on RBC

[0038] In Table 2, starting day 5 after i. v. injection of HuEPO-L-vFc_{γ2}, RBC showed a significant increase when compared to that in the control group (P<0.1). Figure 4 is a graph using data from Table 2.

Table 2. Effect of i.v. injection of HuEPO-L-vFc_{γ2} on RBC (10⁶ cells/μl; ±s, n=3)

Group	Before injection	After injection	Difference
Control	6.11 ± 0.10	6.88 ± 0.60	0.78 ± 0.52
48h	6.10 ± 0.06	7.05 ± 0.30	0.95 ± 0.34
72h	6.10 ± 0.16	6.59 ± 0.23	0.49 ± 0.38
96h	6.09 ± 0.04	7.18 ± 0.54	1.09 ± 0.58
120h	6.04 ± 0.15	7.66 ± 0.30	1.62 ± 0.22*
144h	6.10 ± 0.16	7.35 ± 0.16	1.25 ± 0.02
168h	6.07 ± 0.22	7.47 ± 0.06	1.40 ± 0.27
192h	6.09 ± 0.14	7.84 ± 0.06	1.75 ± 0.09**
216h	6.09 ± 0.10	7.59 ± 0.08	1.50 ± 0.08*
240h	6.08 ± 0.17	8.00 ± 0.26	1.92 ± 0.10**

* P<0.1, ** P<0.05 as compared to the control group.

5.3 The effect of i. v. injection of HuEPO-L-vFc_{γ2} on Hb

[0039] In Table 3, starting 72 h after i. v. injection of HuEPO-L-vFc_{γ2}, Hb showed a significant increase when compared to that in the control group (P<0.1). Figure 5 is a graph using data from Table 3.

Table 3. Effect of i.v. injection of HuEPO-L-vFc_{γ2} on Hb (g/L; ±s, n=3)

Group	Before injection	After injection	Difference
Control	140 ± 2.52	151 ± 8.9	10.7 ± 8.4
48h	134 ± 2.09	158 ± 6.4	24.0 ± 8.2
72h	143 ± 10	158 ± 3.5	15.0 ± 13.2*
96h	139 ± 1.5	167 ± 12.1	28.3 ± 10.7*
120h	141 ± 5.7	176 ± 4.2	35.0 ± 5.0**
144h	139 ± 2.7	166 ± 1.2	27.3 ± 2.5**
168h	140 ± 2.3	170 ± 8.9	29.7 ± 6.7**
192h	141 ± 3.1	183 ± 1.2	42.0 ± 2.0***
216h	139 ± 3.6	172 ± 2.5	32.7 ± 4.0**
240h	139 ± 7.2	185 ± 3.8	45.3 ± 6.0***

* P<0.1, ** P<0.05, *** P<0.01 as compared to the control group.

5.4 Serum levels of HuEPO-L-vFc_{γ2} and pharmacokinetics

[0040] Concentrations of HuEPO-L-vFc_{γ2} in serum samples collected at different time points were analyzed quantitatively by ELISA using anti-HuEPO and anti-human IgG Fc antibodies. Results are shown in Table 4 and also as a graph in Figure 6. Data were also analyzed using the 3P87 software to generate the pharmacokinetic parameters in Table 5. Different time points gave the best fit using a two-compartment model with weight on 1/c. From this calculation, the $T_{1/2\alpha}$ and $T_{1/2\beta}$ of HuEPO-L-vFc_{γ2} in rats are 0.63 h and 14.2 h, respectively. The plasma clearance of HuEPO in rats has also been reported to be nonlinear, often with an initial rapid distribution, followed by a slower elimination phase. The half-life of the β elimination phase has been reported to be about 2 to 3 h (see, for example, Spivak et al., *Blood*, 73:90-99, 1989; Fukuda et al., *Blood*, 73:84-89, 1989; Kinoshita et al., *Arzneimittelforschung*, 42:174-178, 1992). The results of this study indicates that the elimination half-life of HuEPO-L-vFc_{γ2} is much longer than that of rHuEPO, indicating the prolonged presence of the fusion protein in rats.

Table 4. Serum concentrations of HuEPO-L-vFc_{γ2} after injection

Time after injection	Serum Concentration (ng/ml)				Corrected Value
	Rat 1	Rat 2	Rat 3	Average \pm SE	
1 Min.	2989	2654	2282	2641.7 \pm 353.7	2624.5
2.5 Min.	2866	2614	2170	2550.0 \pm 352.4	2532.9
5 Min.	2866	2747	2974	2862.3 \pm 113.5	2845.2
10 Min.	2790	2635	2419	2614.7 \pm 186.3	2597.5
20 Min.	2549	2384	2798	2577.0 \pm 208.4	2559.9
1 Hr.	1244	1545	1694	1494.3 \pm 229.2	1477.2
2 Hr.	1149	1067	1134	1116.7 \pm 43.7	1099.5
4 Hr.	1108	1054	1182	1114.7 \pm 64.3	1097.5
1 Day	250	240	288	259.3 \pm 25.3	242.2
2 Days	103	170	100	124.3 \pm 39.6	107.2

3 Days	98	44	109	83.7±34.8	66.5
4 Days	56	44	63	54.3±9.6	37.2
5 Days	24	26	16	22.0±5.3	4.9
6 Days	30	33	35	32.7±2.5	15.5
7 Days	16	17	15	16.0±1.0	-1.1
8 Days		19	20	19.5±0.7	2.4
9 Days	19		13	16.0±4.2	-1.1
10 Days	22	14	15	17.0±4.4	-0.1

Table 5. Pharmacokinetic parameters after injection of HuEPO-L-vFc₂

Parameter	unit	Value	Standard Error (S.E.)
A	ng/ml	1743. 15	220. 29
α	1/hr	1. 1019	0. 3856
B	ng/ml	1072. 20	207. 27
β	1/hr	0. 04894	0. 00829
V(c)	($\mu\text{g/kg}$) / (ng/ml)	0. 00710	
$t_{1/2\alpha}$	hr	0. 6291	
$t_{1/2\beta}$	hr	14. 1632	
AUC	ng·hr/ml	23490. 4	
CL (s)	$\mu\text{g/kg/hr}$ / (ng/ml)	0. 0085	

$r=0.9927$

[0041] The examples described above are for illustration purposes only. They are not intended and should not be interpreted to limit either the scope or the spirit of this invention. It can be appreciated by those skilled in the art that many other variations or substitutes can be used as equivalents for the purposes of this invention, which is defined solely by the written description and the following claims.